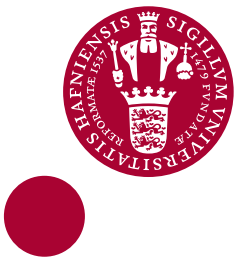

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Published in:
Aquaculture Reports

DOI:
10.1016/j.aqrep.2015.04.001

Publication date:
2015

Document version
Publisher's PDF, also known as Version of record

Aquaculture Reports 2 (2015) 10–16

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

Genetic responses of the marine copepod *Acartia tonsa* (Dana) to heat shock and epibiont infestation

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ARTICLE INFO

Article history:
Received 16 February 2015
Received in revised form 12 April 2015
Accepted 13 April 2015
Available online 24 April 2015

Keywords:
Acartia tonsa
β-Actin
Efa-1α
Epibiont infestation
Ferritin
Heat shock
Hsp70
Hsp90
Gene expression

ABSTRACT

Expression of stress-related genes was investigated in the marine copepod *Acartia tonsa* in relation to heat shock at two different salinities (10 and 32‰), and it was furthermore investigated whether experimentally induced epibiont infestation led to elevated expression of stress-related genes. Expression of the genes ferritin, Hsp90 and Hsp70 were analyzed in adult copepods by conducting reverse transcription-quantitative real time PCR (RT-qPCR). The expression of Hsp70 and Hsp90 was significantly up-regulated after heat shock and the expression levels were higher in copepods cultivated at 10‰ salinity seawater than in copepods cultivated at 32‰. Significant up-regulation of ferritin (3.3 fold increase) was observed as a response to infestation with the epibiotic euglenid *Colacium vesiculosum*. Results suggest that (i) *A. tonsa* responds more pronounced to thermal shock when cultivated in low salinity seawater (10‰) as compared to optimal salinity seawater (32‰) and (ii) epibiont infestation does cause a measureable physiological response in the host.

1. Introduction

Copepods are the dominant zooplankton biomass in the oceans (e.g., Humes, 1994) and copepod nauplii and copepodites constitute the natural food for most marine fish larvae (Hunter, 1981). For this reason, copepods are today cultivated for being used as feed for newly hatched larvae in marine aquaculture systems (Toledo et al., 1999; Støttrup, 2006). Copepods in cultivation systems may be exposed to environmental conditions (e.g., fluctuations in temperature and pH, or pathogens) that challenge successful large-scale production. It is, therefore, essential that copepod species chosen as live feed show high resistance to such conditions. While it is feasible to keep physical and chemical conditions relatively stable in indoor, intensive system, this may be less straightforward in extensive and larger, outdoor production systems. Epibiotic bacteria, protozoans, and algae may appear on copepods in both types of systems. Such epibionts are commonly found on copepods in the wild (Herman and Mihursky, 1964; Møhlenberg and Kaas, 1990; Gerdts et al., 2013) and cannot be completely eliminated in aquaculture systems. It is, therefore, important to evaluate the potential physiological effects of such epibionts.

The calanoid copepod *Acartia tonsa* is a species widely cultivated in research laboratories and it has proven valuable as feed for fish larvae in mariculture (Støttrup et al., 1986; Støttrup and Norsker, 1997). The overall ecology of *A. tonsa* has been studied for decades (Mauchline et al., 1998), but reports on genetic responses to environmental stress in *A. tonsa* have only recently started to appear (Nilsson et al., 2013). For other copepod species, on the other hand, a row of studies have shown that stress-related genes, such as Hsp70 and Hsp90, are expressed at elevated levels as response to, e.g., temperature stress (Voznesensky et al., 2004; Xuereb et al., 2012) and exposure to chemicals (Hansen et al., 2010). Similarly, differential expression of several genes has been explored as response to physiological stage of both adult copepods (Tarrant et al., 2008; Aruda et al., 2011) and eggs (Nilsson et al., 2013). These studies showed that ferritin expression was up-regulated in diapausing copepods (Tarrant et al., 2008) and in quiescent eggs (Nilsson et al., 2013), which is consistent with ferritin being involved in protecting cells from oxidative stress.

Copepods are hosts for a wide range of parasites and epibionts (Théodoridès, 1989; Skovgaard and Saiz, 2006; Skovgaard, 2014), of which epibiotic algae are particularly common. Among the algae associated with copepods, some are endoparasites, whereas oth-

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http://dx.doi.org/10.1016/j.aqrep.2015.04.001

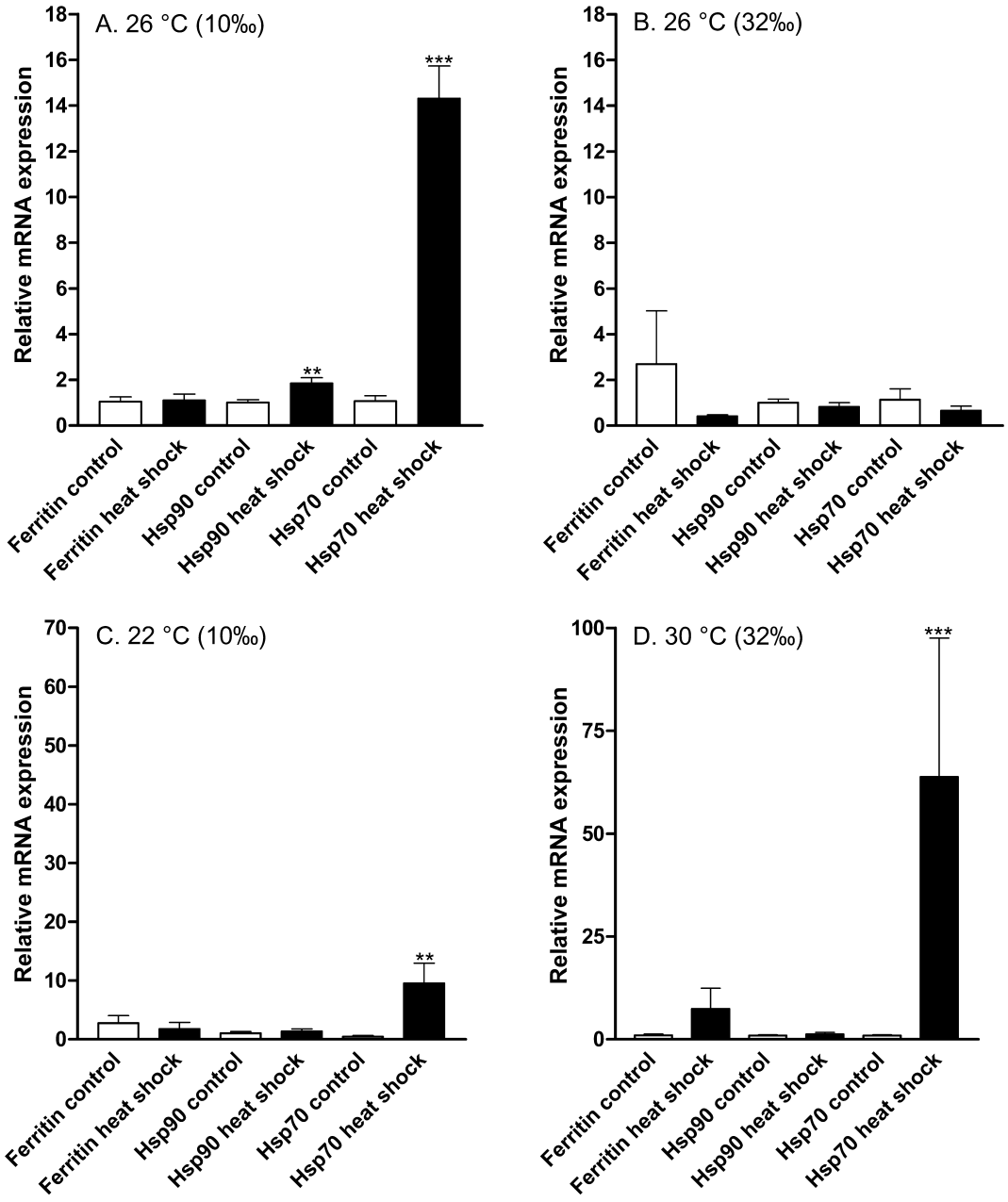

Fig. 1. Heat shock experiments with *Acartia tonsa* incubated for 45 min. Effect on expression of ferritin, Hsp90, and Hsp70 genes as measured by RT-qPCR. Gene expression was normalized to reference genes β-actin and efa-1α. (A) Heat shock at 26 °C, copepods cultivated in 10‰ salinity seawater. (B) Heat shock at 26 °C, copepods cultivated in 32‰ salinity seawater. (C) Heat shock at 22 °C, copepods cultivated in 10‰ salinity seawater. (D) Heat shock at 30 °C, copepods cultivated in 32‰ salinity seawater. Asterisks indicate significant differences compared to the control group, $^{**}p < 0.01$, $^{***}p < 0.001$, $n \geq 5$.

ers are attached to the exoskeleton as epibionts (Michajłow, 1972; Ho and Perkins, 1985). Such epibionts are conceptually often not included in the definition "parasite" (Ebert, 2005), but may still have a negative impact on the host. It is often hypothesized that epibionts have adverse effects on their zooplankton hosts. Studies on epibiont infestation have explored survivorship and reproduction of copepods (Kankaala and Eloranta, 1987; Weissman et al., 1993) and effects of epibiosis on the mating process (Souissi et al., 2013). Infestation with the epibiotic ciliate *Zoothamnium* spp. may increase mortality rate and reduce body size of *A. tonsa* (Burris and Dam, 2014) and, likewise, Visse (2007) concluded the peritrichous ciliate *Epistylis* sp. to shorten the lifespan of *A. bifilosa* females. Similarly, the epibiotic euglenid *Colacium vesiculosum* has been associated with reduced egg production in *A. tonsa* females (Møhlenberg and Kaas, 1990). In addition, epibionts are thought to negatively affect feeding, swimming, and respiration of copepods (Threlkeld et al., 1993). A study on the interaction between filamentous epibiotic bacteria and *Tigriopus brevicornis* showed that epibionts affect copepods' swimming rate (McAllen and Scott, 2000), and Weissman et al. (1993) showed that *A. hudsonica* infested with peritrich ciliates had a significantly slower sinking rate than uninfested copepods. Moreover, studies have revealed indirect effects of epibionts on the host, such as an increased visibility for predators (Willey et al., 1990; Chiavelli et al., 1993). In general, however, effects of epibionts on copepods have been investigated by comparing wild-caught infested copepods with uninfested ones and such studies are likely to be biased because infested copepods are likely to be older, and thereby probably less vital, than the uninfested (Møhlenberg and Kaas, 1990).

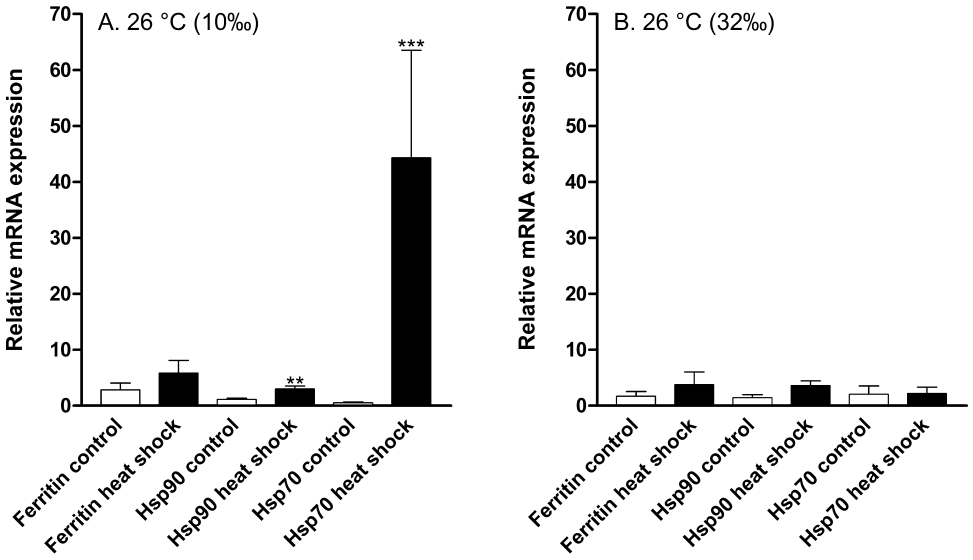

Fig. 2. Heat shock experiments with *Acartia tonsa* hardened at 22 °C and then incubated for 45 min at 26 °C. Effect on expression of ferritin, Hsp90, and Hsp70 genes as measured by RT-qPCR. Gene expression was normalized to reference genes β-actin and efa-1α. (A) Copepods cultivated in 10‰ salinity seawater. (B) Copepods cultivated in 32‰ salinity seawater. The expression level was normalized to β-actin and efa-1α. Asterisks indicate significant differences compared to the control group, $^{**}p < 0.01$, $^{***}p < 0.001$, $n \geq 5$.

The aim of this study was to evaluate expression of the stress-related genes heat shock proteins 90 and 70 (Hsp90 and Hsp70) and ferritin in *A. tonsa* in order to explore whether epibiont infestation leads to detectable changes in expression of stress-related genes under laboratory conditions. The overall methodology was first tested by examining a more straightforward parameter, namely the effect of rapid temperature changes (heat shocks) on copepod stress gene expression and the dependence of heat shock on salinity.

2. Materials and methods

2.1. Cultures

A. tonsa culture was supplied by the National Institute of Aquatic Resources, Danish Technical University, Denmark. The copepod cultures were cultivated at two different salinity seawaters: the original salinity of 32‰ and an additional low salinity of 10‰. The low salinity culture was obtained by gradually diluting half of the original culture with deionized water over a period of two weeks. Copepod cultures were fed three times per week with a unialgal diet of *Rhodomonas salina* cultured in 32‰-salinity f/2-medium (Guillard and Ryther, 1962). A culture of the epibiotic euglenoid *C. vesiculosum* was provided by CCAP (Culture Collection of Algae and Protozoa, Scottish Marine Institute, UK; strain number 1211/3). *C. vesiculosum,* originally grown in freshwater Jaworski's Medium (CCAP), was gradually acclimatized to a mixture of 10‰-salinity f/2 and Jaworski's Medium, reaching a final salinity of 5‰. All cultures were maintained at 15 °C under dim light following a 12:12 h light/dark photoperiod.

2.2. Heat shock experiments

Heat shock experiments were carried out with copepods cultured at 10‰ and 32‰-salinity seawater in 50-mL capacity culture tissue bottles (Greiner Bio-One Austria). Approximately 150 copepods for each salinity (enough for five replicate samples of 20–25 copepods from each bottle) were transferred from 15 °C seawater to 26 °C preheated seawater and then incubated for 45 min at 26 °C in darkness. Secondly, copepods cultivated at 32‰ were exposed to a 30 °C heat shock in a similar manner and copepods cultivated at 10‰ were exposed to a heat shock at 22 °C. Finally, in order to study whether thermotolerance could be induced, copepods (cultivated at 15 °C) were hardened to heat at 22 °C for 45 min. After heat hardening treatment, copepods were exposed to 26 °C for 45 min. This was done for copepods cultivated both at 10‰- and 32‰-salinity seawater. Control groups were in all cases kept at 15 °C, but otherwise manipulated in the same way as the experimental groups.

2.3. Infestation with Colacium vesiculosum

Copepods cultivated in 10‰-salinity seawater were incubated for 24 h in 50-mL capacity tissue culture bottles with and without the addition of *C. vesiculosum* at approximately 2000 cells mL^{-1} (final concentration) and a resulting salinity of 5–10‰. An additional control experiment was performed in which cell-free filtrate from the *C. vesiculosum* culture was added. All treatments and controls were performed with at least 5 replicates of 20–25 copepods at 15 °C under a 12:12 h light/dark photoperiod. Copepods were not fed during the incubation period. Parallel treatments with *C. vesiculosum* were made in order to assess visually whether epibionts actually attached to copepods. Copepods from these bottles were observed and photographed using an Olympus SZX16 stereomicroscope (Olympus, Japan) equipped with a Canon EOS 7D digital camera (Canon, Japan).

2.4. Gene expression analysis

After exposure to heat shock or epibiont infestation, copepods were gently sorted out individually with a pipette and placed in 1.5-mL Eppendorf tubes. Excess water was then removed with a drawn-out Pasteur pipette and 100 μL TRI reagent® (Sigma–Aldrich) was added to the tubes, which were then placed at –80 °C until further analysis.

2.4.1. RNA extraction, purification and cDNA synthesis

The samples filled with 100 μL of TRI reagent® (Sigma–Aldrich, Denmark) were thawed on ice and homogenized, using a disposable plastic micropestle, until no large particles were visible. Samples were vortexed and centrifuged at 10,000 × *g* (at 4 °C) for 2 min.

Each pestle was rinsed with 150 μL of TRI reagent®, which were added to the sample tube. RNA extraction followed the protocol described by Zhang et al. (2013) with minor modifications: The homogenized copepod sample received 1/5 by total volume of chloroform, and tubes were vortexed and centrifuged at 10,000 × *g* (at 4 °C) for 20 min. Approximately 40% of the aqueous layer was transferred to a new 1.5 mL Eppendorf tube and an equal volume of phenol:chloroform was added. Tubes were then vortexed thoroughly and centrifuged at 10,000 × *g* (at 4 °C) for 5 min. This step was repeated twice – or only once if the aqueous RNA-layer was too thin. Less than 40% of total aqueous layer was transferred to a new 1.5 mL Eppendorf tube. RNA purification was conducted according to the manufacturer's instruction (NucleoSpin® RNA Clean-up XS kit of Macherey–Nagel GmbH & Co., KG, Germany) and treated with DNAase I (Fermentas, UK) to remove potential contaminating DNA from the samples. RNA-quality was assessed by measuring absorbance ratios A260/280 and A260/230 of individual samples using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). Purified RNA samples (approximately 300 ng) were converted to cDNA using TaqMan® Reverse Transcription Reagents kit (Applied Biosystems, CA, USA) using an oligo$(dT)_{16}$ primer following the manufacturer's protocol. To dilute potential inhibitors prior to reverse transctription quantitative PCR analyzes, 20 μL of cDNA were diluted 1:10 by adding 45 μL of RNase/DNase free water.

2.4.2. Primer design

Partial sequences of efa-1α and Hsp90 were obtained through PCR amplification of cDNA. PCR primers for Hsp90 were as follows: forward: 5′-CAGCTBAAGGAGTACGACG-3′; reverse: 5′-CTTGGCCGCCATGTATCCC-3′. These primers were designed from conserved regions identified by sequence alignment of Hsp90 genes from seven crustacean species (GenBank accession numbers: EU306564; HQ008268; JF811335; JX624123; GQ227488; KC845247; KF516630). For the elongation factor-1α gene (efa-1α), the RT-qPCR primers given by Lauritano et al. (2011) were used as PCR primers. Hsp90 and efa-1α cDNA was amplified in 25 μL reaction volumes containing 1.25 unit of Biotaq polymerase (Bioline Reagents Limited, London, UK), buffer supplied with the polymerase, $MgCl_2$ at 3.0 mM, dNTPs at 1.6 mM, and the forward and reverse primers at 1.0 mM. The PCR was run in a T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) under the following conditions: initial denaturation for 2 min at 94 °C followed by 35 cycles of 94 °C (15 s), 50 °C (30 s), 72 °C (2 min), and a final extension at 72 °C for 7 min. PCR products were visualized on a 2% agarose gel and selected PCR-products were purified using the 'Illustra GFX PCR DNA and Gel Purification Kit' (GE Healthcare, Little Chalfont, UK) sequenced bi-directionally with an ABI3730xl sequencer (Macrogen Europe, Amsterdam, The Netherlands), using the PCR primers. Finally, sequence reads were aligned and assembled using the software ChromasPro 1.75 (Technelysium, Brisbane, Australia) and RT-qPCR primers were designed for efa-1α (forward: 5′-TCTTAAGCCCGGTATGATCG-3′; reverse: 5′-GAGACTCGTGGTGCATCTCA-3′; amplicon length was 42 bases) and Hsp90 (forward: 5′-GTCACATCCCAGTATGGTTGG-3′; reverse: 5′-CCGTGACACCTCCACCATGG-3′; amplicon length was 78 bases). Primers for Hsp70, ferritin and β-actin were as described by Nilsson et al. (2013).

2.4.3. Reverse transcription quantitative PCR analyzes

RT-qPCR was performed in 12.5 μL reactions using Agilent Technologies Brilliant II SYBR® Green (Sigma–Aldrich) RT-qPCR Master Mix (6.25 μL), 0.5 μL forward primer at 1.0 mM, 0.5 μL reverse primer at 1.0 mM, 2.75 μL RNase/DNase free water and 2.5 μL diluted cDNA template. RT-qPCR was conducted on Stratagene Mx3005P real-time thermal cycler (AH Diagnostics, Denmark) under following conditions: initial denaturation of 95 °C for 15 min followed by 40 cycles of annealing at 95 °C (30 s) and extension at 60 °C (30 s). The program was set to show dissociation/melting curve of each amplicon from approximately 65–70 °C to 90 °C (varied depending on the treatment), and read every 1 °C. Three target genes (Hsp70, Hsp90, ferritin) and two reference genes (efa-1α and β-actin) were analyzed on all samples.

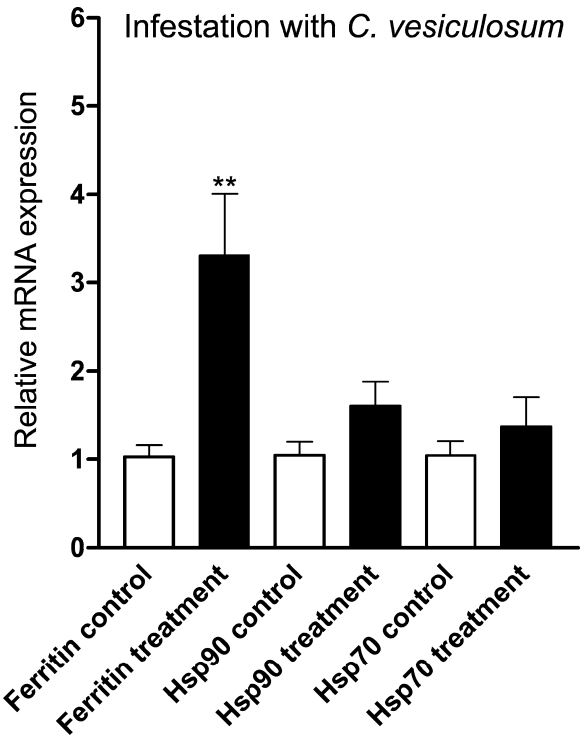

Fig. 3. Exposure of *Acartia tonsa* to the epibiont *Colacium vesiculosum* at approximately 2.000 cells mL^{-1} for 24 h. Effect on expression of ferritin, Hsp90, and Hsp70genes as measured by RT-qPCR. The expression level was normalized to reference genes β-actin and efa-1α. Asterisks indicate significant differences compared to the control group, $^{**}p < 0.01$, $n \geq 5$.

2.4.4. Statistical analysis

To estimate the expression level and to compare to controls and reference genes, the $2^{-\Delta\Delta C_T}$ method was used (Livak and Schmittgen, 2001). Change of threshold cycle number (ΔC_T) was calculated as the difference between C_T values of the target gene and the reference genes for each individual sample. The regulation was considered to be up-regulated or down-regulated when the value of the average was >1 or <1, respectively. Statistical differences between the treated groups were tested using two-sample *t*-test and were considered to be significant when $p < 0.05$. Figures were created using GraphPad Prism 3.0 (GraphPad Software Inc., CA, USA). The results in the graphs are represented as the mean value of the fold change.

3. Results

The expression levels of stress-related genes (ferritin, Hsp90, and Hsp70) were analyzed by RT-qPCR. No down-regulation of gene expression was seen in any of the experiments (Figs. 1 and 2).

3.1. Heat shock (15 °C to 26 °C)

For copepods cultivated in low salinity seawater (10‰), expression of the genes Hsp70 and Hsp90 increased significantly after

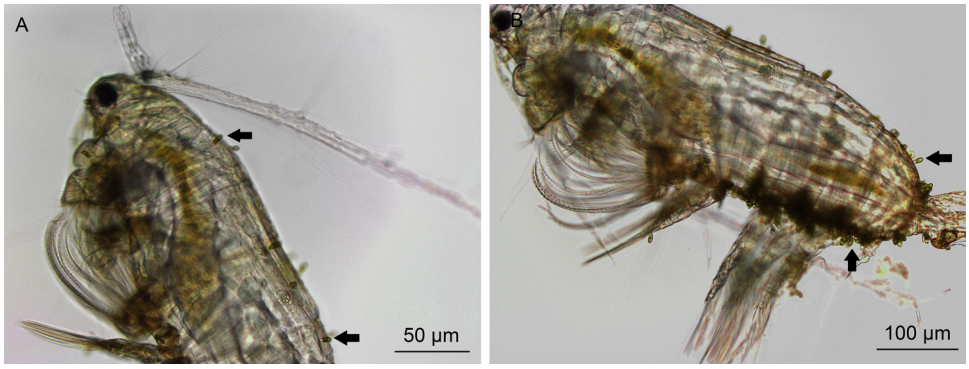

Fig. 4. A and B *Acartia tonsa* experimentally infested with the epibiont *Colacium vesiculosum* (arrows) after a 24-h incubation with *C. vesiculosum* at approximately 2.000 cells mL^{-1}.

45 min at 26 °C compared to control groups, showing 14.3 fold ($p < 0.0001$) and 1.85 fold ($p < 0.01$) up-regulation, respectively (Fig. 1A). The animals appeared to be exhausted after the treatment and did not exhibit the typical escape jumps when being caught with a pipette, as did the copepods in the control group. At optimal salinity seawater (32‰), on the other hand, *A. tonsa* did not respond to the heat shock and did not show differences in expression of any of the genes ferritin, Hsp90 or Hsp70 ($0.22 < p < 0.49$) after exposure to 26 °C for 45 min (Fig. 1B).

3.2. Heat shock (15 °C to 22 °C at 10‰ salinity)

Considering the clear response to a 26 °C heat shock treatment of copepods cultivated at low salinity (10‰), this experiment was repeated at 22 °C. For copepods cultivated at low salinity, a 22 °C heat shock was enough to induce significant Hsp70 ($p < 0.01$) up-regulation (9.58 fold increase) compared to control groups. However, Hsp90 ($p = 0.47$) and ferritin ($p = 0.56$) were not significantly affected by the treatment (Fig. 1C). It was considered needless to perform this experiment with copepods cultivated at 32‰ salinity, since these did not respond significantly to the more drastic heat shock at 26 °C.

3.3. Heat shock (15 °C to 30 °C at 32‰ salinity)

Since no genes responded to a 26 °C heat shock when copepods were cultivated at 32‰ salinity, this experiment was repeated at 30 °C. In this case, a significant up-regulation in the expression of Hsp70 ($p < 0.001$) was seen after exposure to 30 °C for 45 min. The expression for Hsp70 increased to a peak of 63.8 fold (Fig. 1D), while the other two target genes (ferritin and Hsp90) were not significantly affected by the treatment ($p = 0.52$ and 0.24, respectively). This experiment was not performed with copepods cultivated at low salinity (10‰), because at 10‰ copepods appeared exhausted and were almost completely immobile already after the treatment at 26 °C, and further heat stress would probably lead to cell damage and an attempt to measure gene expression would not be meaningful.

3.4. Heat shock after hardening (hardening at 22 °C, heat shock at 26 °C)

After 45 min of hardening at 22 °C, a pronounced up-regulation was observed for copepods cultivated at 10‰ salinity. Hsp70 showed higher up-regulation (44.2 fold increase, $p < 0.001$, Fig. 2A) as compared to the same experiment without hardening at 26 °C (14.3 fold, Fig. 1A) as well as no hardening at 22 °C (Fig. 1C). The same was the case for Hsp90, being up-regulated nearly 3 fold ($p < 0.01$, Fig. 2A). Gene expression in copepods kept in 32‰ salinity seawater (Fig. 2B) did not vary from control experiments (*p* values for ferritin, Hsp90, and Hsp70 were as follows: 0.42, 0.06, 0.48).

3.5. Infestation with the euglenoid epibiont Colacium vesiculosum

For copepods being exposed to and infested with the euglenoid *C. vesiculosum* for 24 h, only ferritin showed a slight, but significant, up-regulation (3.3 fold increase in expression level, $p < 0.01$, Fig. 3). Copepods exposed only to cell-free filtrate of a *C. vesiculosum* culture did not show any significant differences (*p* values for ferritin, Hsp90, and Hsp70 were as follows: 0.4, 0.45, and 0.07; data not shown). After 24 h of incubation, several *C. vesiculosum* cells were attached to each copepod (Fig. 4). It was not possible to observe in detail the copepods used for RT-qPCR, but extra animals were incubated and these were infested by approximately 10–30 *C. vesiculosum* cells attached on the prosome as well as on and around limbs.

4. Discussion

Investigation of physiological responses of copepods at the gene expression level is a discipline that has caught increasing scientific attention during the last decade (Lauritano et al., 2012). However, only few copepod species have so far been subject of such studies and currently a single previous report exists on gene expression in *A. tonsa* (Nilsson et al., 2013).

Previous studies have shown that efa-1α is suitable as reference gene for copepods, such as *Calanus finmarchicus* (Hansen et al., 2008) and *Tigriopus japonicus* (Seo et al., 2006). On the other hand, Lauritano et al. (2011) found that efa-1α was less stable in *Calanus helgolandicus*. In the present study, the two widely used reference genes efa-1α and β-actin were used and their expression was satisfactorily stable in the different treatments of the study (data now shown).

We have shown here that *A. tonsa* adults are able to respond to environment stressors and regulate the stress-related gene expression in response to temperature fluctuations and epibiont infestation. Stress proteins generally have a key role in keeping homeostasis and respond to stress as soon as possible (e.g., Hofmann and Somero, 1996; Helmuth et al., 2002; Tomanek and Somero, 2002). Among various stress-related genes, heat shock protein genes are the most studied, especially Hsp70, which responds to different stressors (Eckwert et al., 1997; Tartarotti and Torres, 2009). For instance, Hsp induction has been shown to be closely related to temperature increase in copepods (Rhee et al., 2009) and other arthropods (Bahrndorff et al., 2009).

The present study showed that induction of heat shock proteins (Hsps) is dependent on both salinity and temperature. Copepods

cultivated at low salinity seawater (10‰) showed higher expression of stress genes after heat shock treatment as compared to copepods cultivated at optimal salinity seawater (32‰). In fact, exposure to 26 °C did not induce elevated Hsps expression in *A. tonsa* cultivated at 32‰ (Fig. 1B), indicating that this exposure was not experienced as stress by the organisms. Hsp90 responded differently than Hsp70 to thermal shock. It was only slightly up-regulated at low salinity (Figs. 1A and 2A), and at optimum salinity (32‰) Hsp90 was unaffected by heat shock even at 30 °C (Fig. 1D). Similarly, expression of Hsp70 in *T. japonicus* is up-regulated after heat shock, whereas Hsp90 expression responds only weakly (Rhee et al., 2009).

When *A. tonsa* was heat hardened at 22 °C, expression of Hsp70 was more pronounced following heat shock at 26 °C (44.2 fold, Fig. 2A) than without hardening (14.3 fold, Fig. 1A). This is in line with typical observations that heat hardening induces thermotolerance by increasing the expression of heat stress-related genes (Bahrndorff et al., 2009). In 32‰ salinity seawater, copepods did not respond to heat hardening (Figs. 1 and 2B), strengthening the above conclusion that *A. tonsa* does not respond to exposure to 26 °C when cultivated in optimum salinity seawater.

It was possible to measure a low, but statistically significant, up-regulation of the expression of ferritin after *A. tonsa* was infested with *C. vesiculosum,* suggesting that epibionts do have a physiological effect on the host. Ferritin is not a general stress protein like the Hsps, but its induction is primarily related to oxidative stress (Orino et al., 2001), and it is overexpressed during bacterial infections and plays a key role in host-pathogen interactions in insects (Kremer et al., 2009; Kosmidis et al., 2014) and fish (Neves et al., 2009). Hence, one may speculate that the up-regulation of ferritin expression in *A. tonsa* was a result of infestation by the photosynthetic epibiont. Levels of heat shock proteins have been shown to increase as results of parasite infection in both vertebrates (Merino et al., 1998) and invertebrates (Rinehart et al., 2002). Nevertheless, neither Hsp70 nor Hsp90 showed significant responses in *A. tonsa* after epibiont infestation. It is possible that infestation load was too low to induce significant Hsp expression. Yet the existing knowledge on copepod stress gene regulation is insufficient for making a qualified prediction of which genes should be up-regulated as result of infestation. So far, only Hakimzadeh and Bradley (1990), using [^{35}S]methionine-labeling and autoradiography, found indications of increased levels of Hsps in *Eurytemora affinis* that was long-term infested with an unidentified algal epibiont. Previous studies have shown it difficult to document negative effects of epibionts through experimental studies (Møhlenberg and Kaas, 1990). However, some authors have concluded epibionts to cause direct adverse effects to copepods (Visse, 2007; Burris and Dam, 2014) and the infested hosts may also be stressed by carrying the additional burden, which changes sinking rate (Allen et al., 1993; Carman and Dobbs, 1997). Souissi et al. (2013) observed that epibiont-infested *E. affinis* males jumped less frequently than epibiont-free males. On the other hand, females did not change their swimming behavior due to infestation. Furthermore, a dense epibiont infestation induced a noticeable mortality rate and reduced fecundity in *E. affinis* (Souissi et al., 2013). Such partial or full castration might be a strategy for epibionts to spread the infection without losing the substrate and resource of nutrients (Barea-Arco et al., 2001; Dubovskaya et al., 2005).

C. vesiculosum cells have been found on copepods and copepodites (Willey et al., 1990). We also observed *C. vesiculosum* cells attached to both *A. tonsa* eggs and nauplii in the cultures, suggesting that epibionts attach to all stages of copepods. Evidence is now building up that copepods are physiologically affected by epibionts, but plausibly the effect will dependent on the physiological stage of the host (Xu and Burns, 1991) as well as the species and number of epibionts.

In conclusion, we found that *A. tonsa* exhibited a significant genetic response to heat shock. This response was more pronounced for Hsp70 than for Hsp90 and the response to heat shock was stronger at low salinity (10‰) as compared to the species' optimum seawater salinity (32‰). Heat hardening resulted in a noticeable increase in the genetic response to heat shock. Finally, experimental infestation with a euglenid epibiont induced a measurable physiological response in the copepod host, which is in line with reports concluding that high epibiont load is likely to exert adverse effects on copepod physiological performance.

Author's contribution

EP and AS designed and performed experiments. All authors contributed to data analysis and writing of the article.

Acknowledgments

AS was supported through the project IMPAQ – IMProvement of AQuaculture high quality fish fry production, funded by the Danish Council for Strategic Research (Grant no. 10-93,522) and AMPHICOP, *Acartia tonsa* Molecular PHysiology – Implementation of novel and fast tools to assess COPepod physiological states, funded by the Villum Foundation (project no. 8960). We are grateful to Birgitte Nilsson for sharing knowledge and methodologies related to copepod RNA work.

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